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## Learning: Classical and Avoidance Conditioning in the Mollusk Pleurobranchaea

**Abstract.** Naive specimens of the marine gastropod *Pleurobranchaea* withdraw from tactile stimulation of the oral veil and show feeding responses to food chemicals. Experimental subjects, trained by pairing touch (conditioned stimulus) with food chemicals (unconditioned stimulus), soon acquired a classically conditioned feeding response to touch alone. Control subjects that received touch alone or unpaired touch and food chemicals showed significantly fewer feeding responses to touch than did experimental subjects. Classically conditioned specimens were used for avoidance conditioning. Subjects that received aversive electrical stimulation when they did not withdraw from touch rapidly learned to withdraw rather than to feed in response to touch alone. Controls that received touch alone or unpaired touch and shock continued to exhibit the feeding response to touch alone. The learned responses persisted for up to 2 weeks without reinforcement before extinction, and could be demonstrated in the isolated nervous system.

The cellular processes underlying learning are largely unknown, in part because suitable experimental preparations have not been developed (1, 2). Such preparations must be capable of learning, and in addition the learned response must be susceptible to analysis on the level of single nerve cells. Gastropod mollusks are attractive candidates for such studies because the neuronal circuitry underlying simple behavioral acts can be comprehensively analyzed in these animals (3) to provide the necessary background information for cellular studies of complex behavioral phenomena (4). Moreover, previous behavioral studies suggest that gastropods are capable of learning (5). We report that the feeding behavior of the carnivorous marine gastropod *Pleurobranchaea californica* can be conditioned, and that the effects of this conditioning can then be detected in the isolated nervous system. This study

demonstrates the first case of associative learning involving a behavior that can be readily studied on the cellular level (6, 7).

Specimens of *Pleurobranchaea* (less than 12 cm long) were maintained individually in white Rubbermaid dishpans (capacity 8 liters) which were

flushed continuously with fresh seawater (2 to 3 liter/min) at ambient temperatures (11° to 18°C). Animals were acclimatized to laboratory conditions for 1 week and then randomly sorted into experimental and control groups. The containers of the two groups were intermingled so that experimental and control animals received the same exposure to light. While in the laboratory, all animals received seawater from a common source and the same quantities of general handling and food (squid twice weekly). Most specimens remained in good physiological condition and gained weight during the experiments.

In the classical conditioning paradigm, experimental animals were trained 20 times daily by stroking the leading edge of the oral veil (Fig. 1A) for 5 to 10 seconds with the tip of a glass probe coated with homogenized squid. Such simultaneous presentation of the conditioned stimulus (tactile stimulation of the oral veil) with the unconditioned stimulus (chemostimulation with natural food substances) elicited the unconditioned response (the feeding behavior) (Fig. 1) in 99 percent of more than 20,000 trials on 65 experimental animals (interstimulus interval, 30 to 60 seconds). In contrast, presentation of the conditioned stimulus alone (tactile stimulation of the oral veil with a sterile glass probe) with the same interstimulus interval caused withdrawal (Fig. 1B) within 3 seconds in 90 percent of 2280 trials on 114 naive specimens. The bite-strike response (Fig. 1F) occurred in only 4.5 percent of these trials, and 53 percent of these responses were produced by seven specimens. The three highest responders were not used in subsequent experiments. These data show that, as required for classical conditioning (8), (i) the unconditioned stimulus (food chemicals) reliably elicited the unconditioned response (feed-

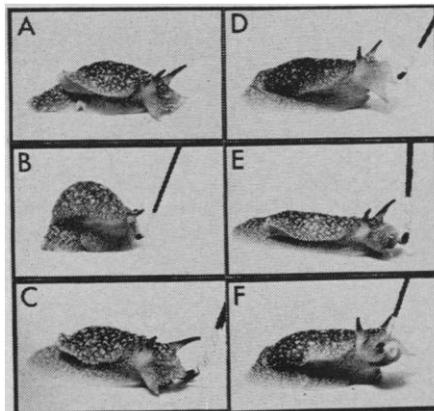


Fig. 1. Behavioral responses of *Pleurobranchaea*. (A) A specimen of *Pleurobranchaea* is shown from the animal's right side; the anterior oral veil and fused lateral tentacles are on the right side of the photograph. (B) A naive specimen withdraws in response to tactile stimulation of the oral veil with a glass probe. The probe is partially wrapped to make it visible for photography. (C to F) Components of the feeding behavior illustrated in a classically conditioned animal are (C) no withdrawal; (D) orientation and following movements; (E) extension of the proboscis; and (F) the bite-strike feeding response. The bite-strike response is fast to distinguish because it happens so fast.

ing); (ii) the conditioned stimulus alone (touch) was ineffective in causing the unconditioned response; and (iii) the animals did not habituate to repeated presentation of the conditioned stimulus at interstimulus intervals employed in the experiments.

Control animals were studied concurrently with experimentals. They received either touch alone or touch followed in 3 to 4 hours by food stimuli, in quantities similar to those for experimental animals. An amount of homogenized squid equivalent to the amount given an experimental animal in 20 conditioning trials was decanted in the vicinity of the oral veil. This chemical stimulus was delivered over an interval roughly equal to the duration of a conditioning session in experimental animals, and induced intense feeding behavior.

Experimental and control animals were tested in a "blind" procedure twice weekly. That is, the tester lacked information regarding the purpose of the experiment and the identity of experimental and control animals. Before test sessions the animals were in-

verted with a sterile probe so that they righted and began locomoting. Tests were made only when the animals were moving smoothly and not exhibiting spontaneous feeding behavior (Fig. 1, D to F). Each animal was tested by 20 presentations of the conditioned stimulus (gentle but firm stroking of the leading edge of the oral veil with a sterile glass probe, with sufficient force to visibly move the oral veil; interstimulus interval, 30 to 60 seconds). All responses were scored within 5 seconds as withdrawal (Fig. 1B) or some component of the feeding behavior (Fig. 1, C to F). In the latter case, tactile stimulation was continued for up to 30 seconds in order to determine how many components of the feeding response could be elicited. To minimize sensitization of the feeding response (2), none of the animals were fed nor presented with food stimuli during the 18 hours before testing.

Figure 2A shows the results of one classical conditioning experiment with 17 specimens. Experimental animals, subjected to classical conditioning for 7 days, showed a progressive increase in

the frequency of the conditioned bite-strike feeding response in successive test sessions, and the conditioned response persisted for several days following the cessation of conditioning. Control animals, subjected to unpaired presentation of the conditioned stimulus (touch) and the unconditioned stimulus (food chemicals) for 7 days, also showed an increase in the feeding response to touch alone, but after 1 week of conditioning the response frequency of experimental animals was invariably significantly higher than that of controls (Mann-Whitney U test,  $P < .02$ ). The lower feeding frequencies of the controls did not result from an inability of these animals to learn, for controls subsequently responded readily to classical conditioning (days 9 to 14 of Fig. 2A). We conclude that the temporal pairing of touch with food chemicals was responsible for the large increase in frequency of feeding response to touch shown by experimental animals. The moderate increase in the response frequency of the control group may represent either long-term sensitization of the feeding response or learn-

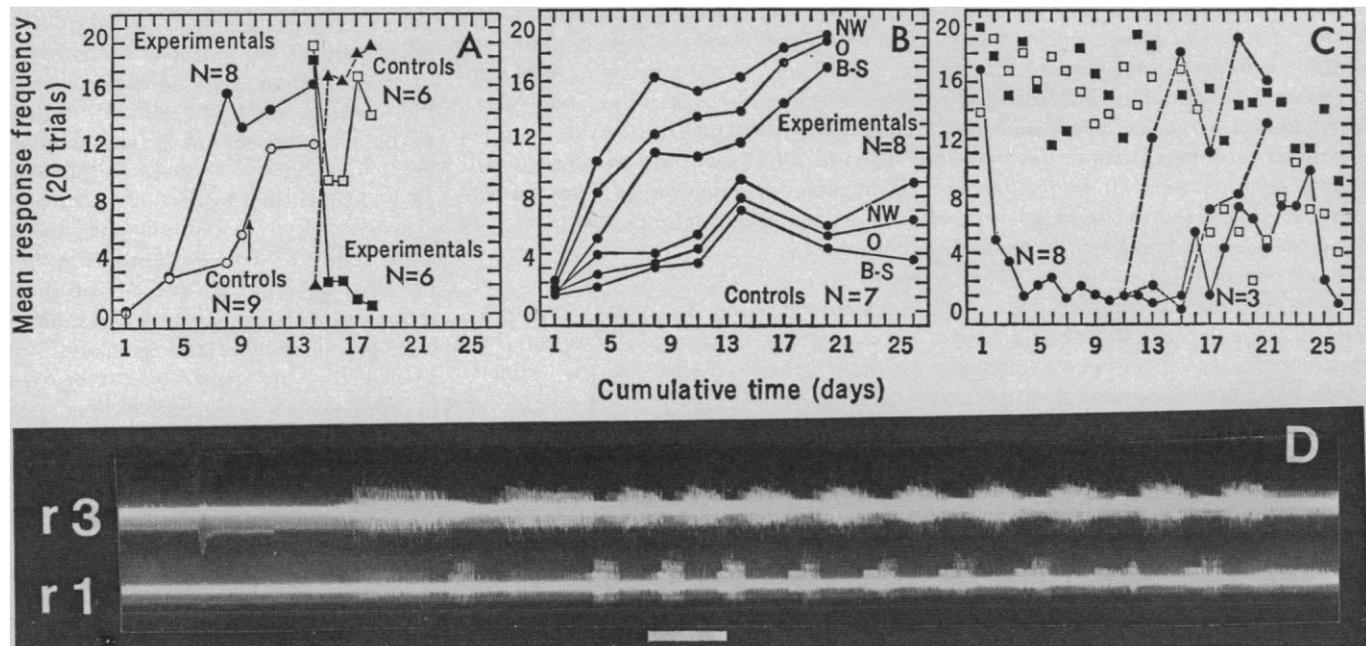


Fig. 2. Conditioning of feeding and withdrawal behavior in *Pleurobranchaea*. (A) A classical conditioning experiment involving the bite-strike response was done on days 1 to 14. Arrow designates beginning of classical conditioning of control animals. Avoidance conditioning was done on days 15 to 18; ■ and □ denote bite-strike responses of experimentals and controls, respectively, and ▲ denotes withdrawal responses of experimentals. (B) A classical conditioning experiment is shown; NW, no withdrawal; (O), orientation; and (B-S), bite-strike. Conditioning was stopped on day 13. (C) Feeding responses during avoidance-conditioning experiment are shown; ●, experimental animals (initially eight specimens); □, controls given unpaired shock and touch (four specimens); and ■, touch controls (three specimens). On day 11, one experimental animal was returned to the classical conditioning paradigm (● and dashed lines); two more were used as touch controls and on day 15 were also returned to classical conditioning (● and solid lines). Avoidance conditioning of the remaining three experimental animals was stopped on day 15 (● and solid lines). The subsequent behavior of the experimental animals reflects extinction first of the avoidance response (days 15 to 24) and then of the bite-strike feeding response (days 24 to 26) that had been classically conditioned previously. (D) Rhythmic motor output obtained from feeding nerves (roots 1 and 3 of buccal ganglion) in the isolated nervous system of a classically conditioned specimen. Sustained stimulation (10 sec<sup>-1</sup>) was given to the tentacle nerve throughout the recording. Naive preparations tested concurrently under the same conditions showed at most three output cycles. Time mark, 10 seconds; r, root.

ing based on pairing of touch with sensory feedback from the few feeding movements elicited during testing.

Animals classically conditioned as described were then divided at random into experimental and control groups for avoidance conditioning. In daily sessions each experimental animal was presented with 20 tactile stimuli to the oral veil (interstimulus interval, 60 seconds). If the animal failed to withdraw within 5 seconds while being stimulated with touch, it received a train of aversive electrical shocks on the oral veil (30 to 50 volts, 10 per second for 1 to 3 seconds). The animal could avoid the shocks by withdrawing initially. Shocks were delivered either by means of two electrodes that spanned the oral veil without contacting it (experimentals in Fig. 2A and all controls), or by means of electrodes wrapped around the test probe and brought into direct contact with the oral veil (experimentals in Fig. 2C). In all cases the same region of the oral veil was stimulated, as indicated by localized dimpling of the skin, and evidence of effective shock was rapid and complete withdrawal of the animal.

Control animals were studied concurrently with experimentals. Each control animal received as many electric shocks as the mean number of shocks delivered to experimental animals, followed in 2 to 3 hours by tactile stimulation in connection with testing. Thus, control animals received unpaired shock and touch. Control animals were tested daily with 20 presentations of touch alone (interstimulus interval, 60 seconds). True "blind" testing was not possible in the avoidance conditioning experiments, because the delivery of electric shocks to experimental animals was contingent on the observed response to touch. Insofar as possible, however, experimental bias was avoided by concealing from the tester both the previous history of each animal and the goal of the experiment.

As a result of avoidance conditioning, animals rapidly developed withdrawal responses to touch alone, accompanied by a decline in the classically conditioned feeding response (Fig. 2A). In contrast, control animals retained low frequencies of withdrawal response to touch alone and showed little decline in the frequency of the feeding response (Fig. 2A). The experimentals were significantly different from controls in the fourth and fifth test sessions (Mann-Whitney U test,  $P < .05$ ). We conclude that the temporal pairing of touch with

shock was responsible for the increase in the withdrawal response frequency and the large concomitant decline in the feeding response frequency of experimental subjects. The depressed feeding rate was not caused simply by short-term shock-induced inhibition (9), because it persisted for several days in the absence of electrical shock (Fig. 2C, days 11 to 15, lower dashed curve).

The results described have been replicated and extended in different experiments (Fig. 2, B and C) (10). Figure 2B shows that classical conditioning included not just the bite-strike response (Fig. 1F), but also the other components of the feeding behavior (Fig. 1, C to E). Moreover, the mean latency of the bite-strike response decreased monotonically with training, from 10.5 seconds for experimentals in the second test session of Fig. 2B to 3.5 seconds in the seventh test session. The control animals, which received only the conditioned stimulus (touch) in this experiment, showed a moderate increase in the frequency of the feeding response to touch; that is, the means in different test sessions were significantly different (Friedman two-way analysis of variance), but the experimentals were significantly different from the controls at  $P < .02$  in all test sessions but the fifth, in which experimentals differed from controls at  $P < .065$  (Mann-Whitney U test).

Figure 2C shows the results of an avoidance conditioning experiment in which unpaired shock and touch controls and touch controls (defined above) were studied concurrently with experimental animals. The experimentals again showed an immediate decline in the frequency of the feeding response. This decline was not caused by a progressive inability of the experimental animals to show the classically conditioned bite-strike response, because when they were returned to the classical conditioning paradigm (Fig. 2C), they rapidly relearned to produce the bite-strike response to touch alone. The acquisition rates were now greater, signifying retention of learning from the previous classical conditioning. In contrast, control animals showed no decline in the frequency of the bite-strike response until the end of the second week without reinforcement. We attribute the decline then to extinction of the classically conditioned feeding response acquired previously.

Having demonstrated associative learning in intact specimens of *Pleurobranchaea*, we sought evidence of learn-

ing in the isolated nervous system. Alternating discharge of motoneurons supplying antagonistic feeding muscles can be recorded from nerves of buccal ganglia in isolated nervous systems (7). In 25 naive animals, electrical stimulation of the nerves that connect the oral veil and tentacles with the brain was ineffective in causing sustained feeding output (7). In the present experiments, these same nerves, which transmit the conditioned and unconditioned stimuli to the brain, were electrically stimulated in isolated nervous systems of four naive, two avoidance-conditioned, and six classically conditioned animals in double-blind experiments. That is, one person dissected the nervous systems from the animals and prepared them for the experiments, and a second person performed the electrophysiological tests; the past history of the animals was concealed from both participants, and the purpose of the experiment was withheld from the dissector. Stimulation of brain roots in the four naive and two avoidance-conditioned animals yielded the same weak efferent feeding discharge obtained previously from naive animals. In contrast, electrical stimulation of the brain roots of three of the six classically conditioned specimens yielded strong, rhythmic feeding output which could be sustained for many output cycles (Fig. 2D) and repeatedly elicited for up to 24 hours. We interpret this result to mean that the afferent pathways contained within the stimulated brain roots became more efficacious in eliciting the feeding rhythm as a result of conditioning. These initial electrophysiological results encourage the view that the cellular basis of associative learning can be explored further in *Pleurobranchaea*.

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10. At ambient seawater temperatures of 15° to 18°C (June to September), all classically conditioned individuals that remained in good physiological condition, that is, continued to show vigorous unconditioned feeding responses, exhibited learning. Of five attempts to replicate the classical conditioning experiment, our single failure (15 experimentals and 15 controls) was performed in November, when the ambient seawater temperature was 11°C and the physiological condition of the animals was deteriorating for unknown reasons. Of five attempts to replicate the avoidance conditioning, all succeeded, including one experiment performed at 11°C.
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## Selective Visual Experience Fails to Modify Receptive Field Properties of Rabbit Striate Cortex Neurons

**Abstract.** *During development, rabbits were exposed only to vertical or horizontal lines to determine if the receptive field characteristics of visual cortex cells would be altered as they are in the cat. Motion and directional selectivity were preserved, and orientation specificity remained unaffected by the restricted experience, which suggests that the rabbit may lack the neural plasticity seen in some other mammals.*

It is well known that modifications of the visual environment can produce dramatic alterations in the brain. Enrichment of visual experience as well as visual deprivation have been shown to cause corresponding changes in the anatomy, physiology, and behavior of some mammalian species (1-3). Recently, it has been demonstrated in the cat that the type of visual experience to which that mammal has been exposed during development may actually determine the receptive field properties of single neurons in its visual system (4, 5). Most single cells in the cat's visual cortex are responsive only to straight line stimuli such as edges or bars, and these must be of a specific orientation. In the normal animal, the optimal orientation of such stimuli varies from cell to cell, so that all axes of orientation are represented (6). When the visual experience of kittens is restricted to vertical or horizontal stripes, the neurons in the visual cortex of these animals will respond only to bars whose orientation is similar to that experienced during infancy (4, 5). These experiments suggest that the visual environment can influence the functional neural connections of at least one species.

The mechanisms which permit these neural modifications in the cat have not yet been specified, although phylo-

genetic status or type of visual system organization may in part determine the extent to which a given species is capable of such alterations. These considerations would seem to be of par-

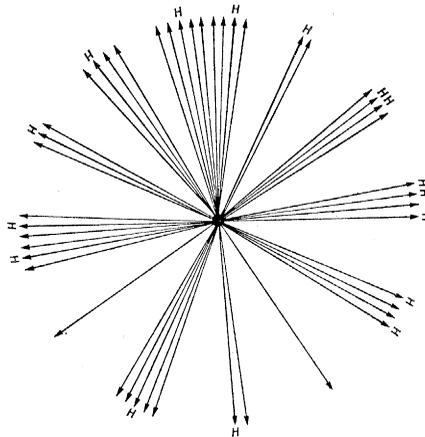


Fig. 1. Preferred directions of 45 directionally selective single cells recorded from the striate cortex of nine rabbits. Directional preference is indicated by the arrow and is related to the animal's visual field. Cells recorded from rabbits exposed to horizontal stripes are designated by an H at the arrowheads. All others were recorded from vertically experienced animals. Directional preference was always classified in 30° axial steps. Arrows have been equally distributed around the 30° axes for clarity only. Top, superior; bottom, inferior; left, anterior; right, posterior.

ticular relevance in the light of evidence that the formation of neural connections in some lower vertebrates cannot be appreciably modified by visual experience (7). Thus, the degree of encephalization of visual function, the extent to which the retinal projection is crossed, the amount of binocular interaction present, or the complexity of visual processing which occurs at the level of the retina may all relate to the degree of neural plasticity possible in the visual system of different animals.

The rabbit visual system provides an excellent opportunity to explore the relation of these factors to plasticity in the nervous system, since the organization of this visual system combines properties seen in both lower vertebrates and higher mammals. As with the visual systems of higher mammals, a high degree of encephalization of visual function is present in the rabbit. On the other hand, the visual system of the rabbit parallels that of certain lower vertebrates to the extent that complex analysis of specific stimulus features occurs at the level of the retina.

We have found that receptive field properties of single neurons in the primary visual cortex of the rabbit are not modified by selective visual experience. Restriction of visual experience to stripes of a particular orientation fails to produce changes in the orientation preferences of orientation-sensitive cortical cells, and neither motion nor directionally selective cells, also found in the visual cortex of the rabbit, are altered by the selective environment. Our findings suggest that the capacity for visual system reorganization imposed by selective visual environments may vary dramatically among various mammalian species.

We reared Dutch belted rabbits in the dark from shortly after birth, and at about 10 days of age, when their eyes normally open, we began placing them in "visual experience" tubes which replicated as precisely as possible those previously used in kitten studies (5). Each tube was constructed of molded plexiglass 92 cm high and 46 cm in diameter with black and white stripes of different widths placed all around the perimeter of the tube. Some of the tubes had horizontal stripes, some vertical. Each tube was illuminated by a photoflood lamp whose light was evenly diffused through a translucent plexiglass lid which covered the top of the tube.

On each test day, the rabbits were placed on a clear plastic disk in the